The emitting state of tryptophan in proteins with highly blue-shifted fluorescence

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The Emitting State of Tryptophan in Proteins With Extremely Blue Fluorescence

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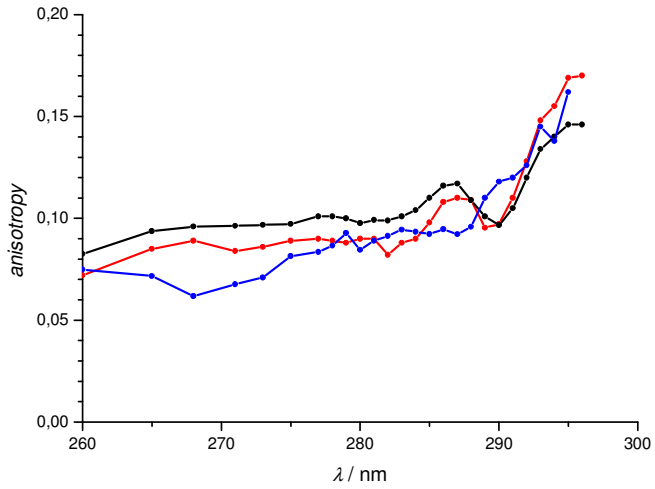

Figure S1. Excitation anisotropy spectra at 15°C of apo-azurin in 60% (v/v) PG (black) and of Wt dI (red) and dI.M97V (blue) in 70% (v/v) glycerol. The emission was collected at 305 nm.

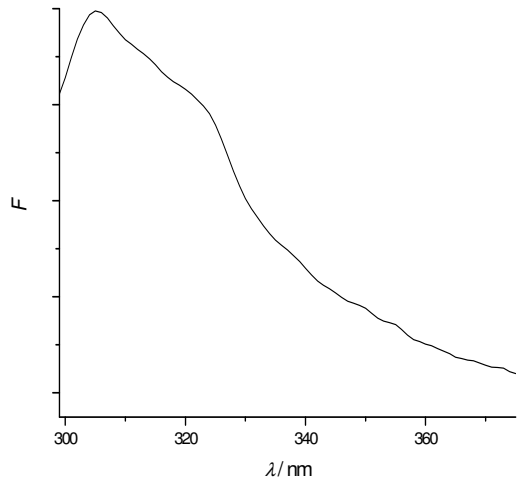

Figure S2. Emission spectra dI.M97V in buffer, pH=7.4, at room temperature. Excitation was at 292 nm.

Experimental Section

Apo- and Zn azurin were isolated as described.[1,2] The emission spectra of Cu^{2+} or Zn^{2+} holo azurin are essentially identical to apo-azurin.[1] Zn^{2+}-azurin (previously known as azurin*)[1] was used because Trp48 is largely quenched by Cu^{2+}. dI and dI.M97V were isolated as described.[3]
Fluorescence experiments were performed essentially as described in detail elswhere.[4] For spectra at low temperature the samples contained 10 mM sodium phosphate buffer, pH=7.4, 30 µM protein or NATA and 90% (v/v) PG (NATA), 60% (v/v) PG (azurin), or 70% (v/v) glycerol (dI). Spectra were recorded on a Fluorolog3-22 fluorospectrophotometer (Jobin Yvon). Unless stated otherwise, excitation and emission bandpasses were set at 2 and 5 nm, respectively. Excitation spectra were corrected using a function supplied by the manufacturer. Emission spectra were corrected using a function derived from a comparison of the recorded spectrum and the published spectrum[5] of a solution of tyrosine (Across, 99+%). The reproducibility of the anisotropy values was found to be ± 0.01.

Details of the QM-MM simulations

Azurin: We have performed QM-MM simulations starting with the X-ray structure for azurin Pae (4AZU) solvated in a 30 Å radius sphere of explicit TIP3 water. Methods and procedures follow closely those of Vivian and Callis[6] except that a quantum mechanical calculation was made after each 1 fs step. Three 10-ps trajectories were averaged, each starting after a different equilibration time of 1, 10, and 30 ps. Each trajectory began with the Trp in the ground electronic state. After 50 fs, the system was put in the ^1L_a excited state.

Transhydrogenase dI: The crystal structure (1F8G) was modified by changing all Se-Met to Met and solvated in a 35 Å radius sphere of explicit TIP3 water. Because of the size of dI, the 35-Å sphere did not enclose the entire protein; it was centered over the domain containing the Trp, and the protein outside the water was held fixed during the MD simulations.

Transhydrogenase dI.M97V: The 1F8G structure was modified to the M97V mutant using spdbv [http://expasy.org/spdbv/], which provided 9 rotamers (3 variants of 3 nominal rotamers) of the Val97 sidechain. The previously used procedure for solvating the protein[6] inserts a water in cavities within the protein if the oxygen is no closer than 2.6 Å. This resulted in a water in close contact with Trp72 in the wt, and two such waters in the mutant. These waters were in a completely hydrophobic pockets and were not among the many observed in the crystal structure nor in related structures of dI. We therefore chose to delete these waters for both the wt and mutant proteins. Further motivation for this choice is that agreement with experiment was much improved; these waters typically created a large red shift of ^1L_a for dI relative to azurin, and for the M97V mutation relative to wt, both in disagreement with experiment. Because the crystal structure was altered, longer (50 ps) simulations were performed for dI, one with the close water and another without.

Distinguishing ^1L_a and ^1L_b: As before[6], the two low lying states were distinguished using the projection of their one-electron transition densities onto an idealized ^1L_a transition density. In the trajectories of Figs. 4a, 4b, and S3, about 5% of the points were omitted because no state had a projection exceeding 0.5.

Comments on results of the QM-MM simulations

For azurin, we found that the protein environment stabilizes ^1L_a by about 750 cm^{-1} more than it stabilizes ^1L_b. This is a clear prediction that ^1L_a will be the fluorescing state. In vacuum, two-photon spectra[7] place the ^1L_a origin of 3MI within 500 cm^{-1} of the ^1L_b origin; for 3MI in solid argon, the gap narrows to only 250 cm^{-1} because ^1L_a is more polarizable than ^1L_b. This red shift due to dispersion interactions with non-polar groups is not included in the simulations; only electrostatic effects are included. Empirically adding this extra 250 cm^{-1} shift, the simulations predict that the ^1L_a origin will lie ~500 cm^{-1} below the ^1L_b origin in the protein. This is nearly 2.5 kT at room temperature, so that little emission from ^1L_b is expected. Expressed as λ^{max}, the average red shift for ^1L_a relative to vacuum is found to be about 8 nm.

It is not possible to attribute this net shift to any one residue because the largest individual contributions come from charged groups on the surface that are in line with the long axis of Trp48 and typically lie within 12-18 Å. Excitation to ^1L_a causes a shift of electron density from the pyrrole to the benzene ring of Trp, meaning that

negative charges near the pyrrole end and/or positive charges near the benzene end shift the emission to longer wavelengths (red shift), and vice versa for a shift to shorter wavelengths. In azurin the distribution of the charged groups is extremely even, such that the *average* total red and blue shifts from charged residues are both ~40 nm, thereby canceling to a net shift on the order of 1 nm. Bulk and nearby water also contributes an average ~1 nm shift. The net average red shift therefore appears to come mostly from a slight bias arising from the uncharged polar groups and the protein backbone. Note that there are ± 5 nm fluctuations in the net shift during the course of a simulation.

For wt dI, a typical trajectory is shown in Figure S3. The prediction from two 50 ps simulations is that the ^1L_a origin lies just below that of ^1L_b and this simulation also predicts a slight blue shift of λ^{max} relative to azurin, in disagreement with the observed 1.5 nm red shift. As for azurin, in dI there is also massive cancellation of contributions from the charged groups. However, in dI, the charged groups contribute about half of the shift, with lysines and arginines together contributing a net red shift of about 20 nm and aspartates and glutamates contributing a net blue shift of about 5 nm. Bulk water contributes a blue shift of ~8 nm. The largest individual contributor is Lys25 with an 8 nm red shift, but the sum of all red shifts is 38 nm from a total of 32 groups, with most contributing less than 1 nm.

For the M97V mutant, all but one of the 9 50-ps simulations predict a slight blue shift (~2 nm) for the ^1L_a fluorescence relative to wt dI, in agreement with experiment. The average stabilization of ^1L_a relative to ^1L_b is only 315 cm^{-1}, with a standard deviation of 100 cm^{-1}. Using the reasoning applied to azurin and wt dI (see above), this predicts ^1L_a to be only ~50 cm^{-1} below ^1L_b, in accord with our observation of a substantial component of ^1L_b emission for dI.M97V. (The Boltzmann factor for the 50 cm^{-1} gap at 288 K suggests that 44% of the molecules will emit from ^1L_b .)

One might well ask why the proteins studied in this work have such short λ^{max} values compared to several other proteins whose Trp residues are also buried in hydrophobic environments. Our best answer at this point is that the net shift is produced by near cancellation of a multitude of fairly large electrostatic contributions from charged and polar groups, including bulk and nearby water. Statistically, it is improbable for these to nearly cancel. We have noted before a surprising bias for protein contributions to be red shifting in a sample of 24 single-Trp proteins.[6] Mutation of the mostly non-polar methionine group to a valine in dI.M97V probably creates a myriad of small displacements of charged and polar groups. One must also consider the loss of the polarizable sulfur, which lies near the Trp72 HN. The likely loss of an induced dipole in the sulfur atom from the polar HN bond is not considered in our purely point charge electrostatic model, but it may be a significant contributor to the blue shift accompanying the M97V mutation.

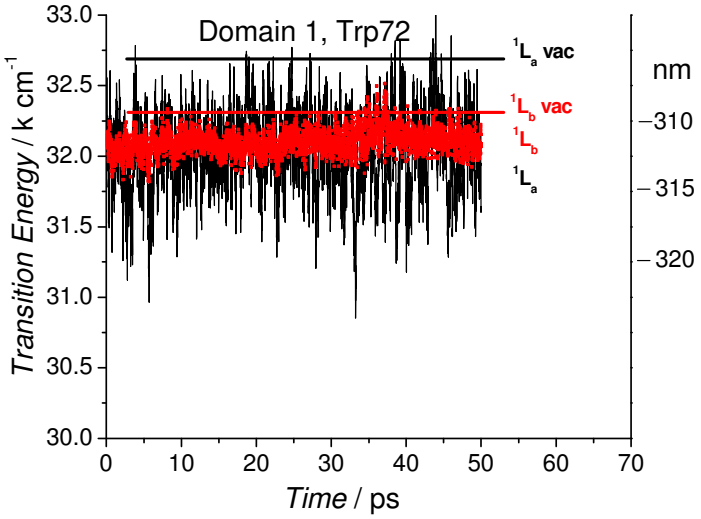

Figure S3. Simulated ^1L_b (red points) and ^1L_a (black line) transition energies for Trp72 of wt dI. In the absence of simulated environmental electrostatic interactions, the calculations give transition energies given by the horizontal lines.

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